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RESEARCH ARTICLE

Spectrophotometric Method for Determination Folic Acid via Diazotization and Coupling with 4-Aminoantipyrine

Resala A. Saleh[1*], Sarmad B. Dikran[2]

Chemistry Department, College of Education for Pure Science (Ibn Al-Haitham), Baghdad University, Baghdad, Iraq.

***Corresponding Author: Resala A. Saleh**

Abstract

Uncommon coupling reagent is used for the simple and sensitive spectrophotometric determination of folic acid in pure form and in its pharmaceutical preparations. The method is based on the diazotization of the p-aminobenzoyl glutamic acid formed after reductive cleavage of folic acid, followed by coupling 4-amino antipyrine to give an orange product with λmax of 469nm. The results follow 'Beer's law in the concentration range of 2-100 $\mu g.mL^{-1}$ with molar absorptive of 3266.36 $L.mol^{-1}.cm^{-1}$. The limit of detection was found to be 0.1482 $\mu g.mL^{-1}$ and the Sandell's sensitivity value was 0.1351 $\mu g.cm^{-2}$. Common excipients used as additives in pharmaceutical preparations do not interfere in the proposed methods. The proposed method was successfully applied to the determination of folic acid in pharmaceutical preparations.

Keywords: *Folic Acid; Spectrophotometry; 4-Aminoantipyrine*

Introduction

Vitamins are a group of compounds principal for the normal development and concrescence of organisms. Folic acid (FA) and folates, which are different chemical forms of vitamin B9, belong to the water-soluble B-group vitamins [1]. Folate is a universal term for compounds possessing vitamin activity similar to that of pteroylglutamic acid and is the form of the vitamin naturally existent in foods [2].

Folic acid is a synthetic form of folate, found in vitamin supplements and reinforced foods [3]. The prime dietary sources of folic acid are spinach, white beans, asparagus, dark-leaved vegetables, Brussels sprouts, soybean, and its derivatives, oranges, and melons, amongst others [4].

At present, vitamin deficiency results mainly from poverty, indigence, food preferences, drug use, and chronic addiction alcohol, amongst other causes. If vitamin intake insufficient, multivitamin preparations can be used in order to prevent vitamin deficiency and the associated physiological problems [5]. Perfect reviews and treatises concerning diverse aspects of folic acid have been published. A survey of the literature reveals that there are various methods available for the determination of folic acid which include social methods [6, 7], liquid chromatography [8,9], high-performance liquid chromatography [10, 11], radio assay [12], histochemical [13], flow-injection chemiluminometry [14], fluorimetry [15], electroanalytical techniques [16, 17], and spectrophotometric methods [18, 20]. Figure (1) shows the structure of folic acid ($C_{19}H_{19}O_6$) [21].

Most of the spectrophotometric methods reported already suffer from some of disadvantages such as narrow range of determination require heating or extraction, long time for the reaction to complete, and instability of the colored product formed.

The idea of the present work is to provide simple, sensitive, and rapid spectrophotometric methods for the determination of folic acid in pure form as well as in folic acid tablets. The outstanding feature of the methods is that all the methods are free from interference when excipients are present, particularly some vitamins.

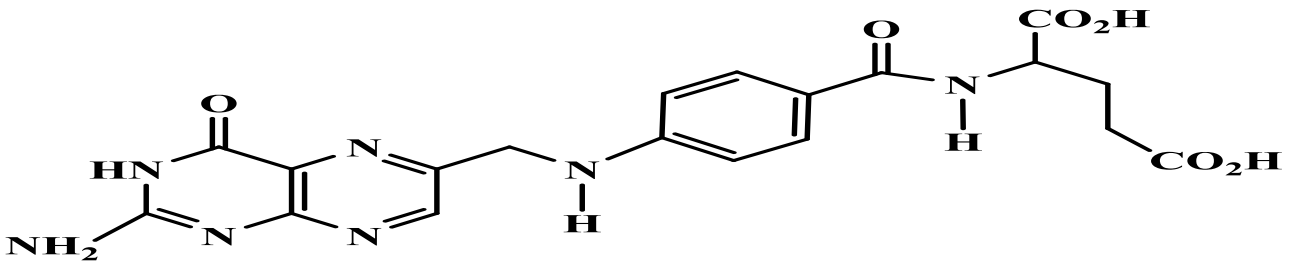


Figure1: Structure of the folic acid molecule

Methodology

Apparatus

All spectrophotometric measurements were performed using Shimadzu 1800 UV-Vis; with match silica cells, a Sartorius BL 210S balance, hot plate with a magnetic stirrer (Germany) and water bath (Memmert W-200 RING- Germany) were used throughout the work.

Materials

All chemicals and reagents used were of analytical grade. Folic acid and 4-amino antipyrine were received as a powder in pure forms as a gift sample from the State Company for Drug Industries and Medical Appliances Samara-Iraq (SDI). Sulfuric acid (GCC) and sodium hydroxide were supplied from Riedel-de Haën, sodium nitrite (BDH), potassium hydroxide (Panreac). Commercially available pharmaceuticals MED-Folic acid (Lebanon), Fulcium (U.A.E), FoliGuard (Bulgaria-EU) and folic acid (UK) were obtained from local markets.

Preparation of Stock and Working Standard Solutions

- A stock solution of (1000μg.mL^{-1}) of folic acid was prepared by dissolving accurately 0.1g of the compound in 20mL of 0.2M sodium hydroxide solution. This solution was reduced using 0.14g of zinc powder in 10mL of concentrated hydrochloric acid (11.8M) solution, filtered, and diluted to 100mL in a calibrated flask. The stock solution was further diluted to get working concentrations.

- 2% (w/v) solution of 4-amino antipyrine was obtained by dissolving 2g of this compound in 100mL in distilled water.

- Approximate solutions of 0.4% sodium nitrite, 1.85M sulfuric acid, 4M sodium hydroxide, 4M potassium hydroxide were prepared by dissolving the required amounts of each reagent in an appropriate volume of distilled water.

Preparation of Sample Solution

A weight equivalent to ten tablet (0.7428g, 1.3760g, 1.1358g, 1.6096g and 0.5866g) was taken from the fine powder of 20-tablets of the pharmaceutical preparations folic acid (5mg/tablet) (SDI), MED-Folic acid (5mg/tablet) (Lebanon), Fulcium (5mg/tablet) (UAE), foliGuard (5mg/tablet) (Bulgaria-EU) and folic acid (5mg/tablet) (UK) respectively and dissolved individually in 20mL 0.2M of sodium hydroxide solution. These solutions were reduced using 0.14g of zinc powder in 10mL concentrated hydrochloric acid (11.8M), filtered, and diluted to 100mL in calibrated flasks.

General Procedure for the Determination of Folic Acid

Under Condition Established by the Univariate Method

To a series of 10mL volumetric flasks, 1 mL aliquots of a standard solution containing (2-100) μg.mL^{-1} of folic acid were transferred. To each flask was then 0.2mL of 4% $NaNO_2$ and 0.7mL of 1.85M H_2SO_4 solutions were added with shaking. Then after, the flasks were placed in an ice bath (0-5) ^{0} C for 5min. 0.4 mL of 2 % (w/v) 4-aminoantipyrine reagent solution was then added and the flasks were allowed to stand in a water bath for 1 minute at 85 ^{0} C. Finally, before diluting the solutions to their final volume with distilled water, to each flask 1mL of 4M KOH solution was added and each mixture was left for 30 sec. in an ice bath. The value of the absorbance of the reaction product was measured at 469.0 nm against reagent blank.

Under Condition Established by Design of the Experiment

To a series of calibrated 10mL volumetric flasks containing 1.0 mL aliquots of standard folic acid solution in the concentration range of (2-100 μg.mL^{-1}), 0.2 mL of 4 % (w/v) $NaNO_2$ and 0.7 mL of 1.85M H_2SO_4 solutions were added with shaking. After maintaining the flasks at (0-5) ^{0} C for 5min. in an ice bath, to

each flask 0.32 mL of 2 % (w/v) 4-aminoantipyrine solution was added and the flasks were allowed to stand in a water bath for 6.2 minutes at 85 ^{0} C. Then after, 1.32mL of 4M KOH was added to each flask and the flasks were left for 30 sec in an ice bath (0-5) ^{0} C before dilution to the final volume with distilled water. The value of the reaction product absorption was measured against the reagent blank at 467.0 nm.

Results and Discussion

Selection of Wavelength

The absorption spectra of the product solution versus reagent blank and for reagent blank versus distilled water were recorded (Fig. 2). The product shows maximum absorption at 469.0 nm under the primary test.

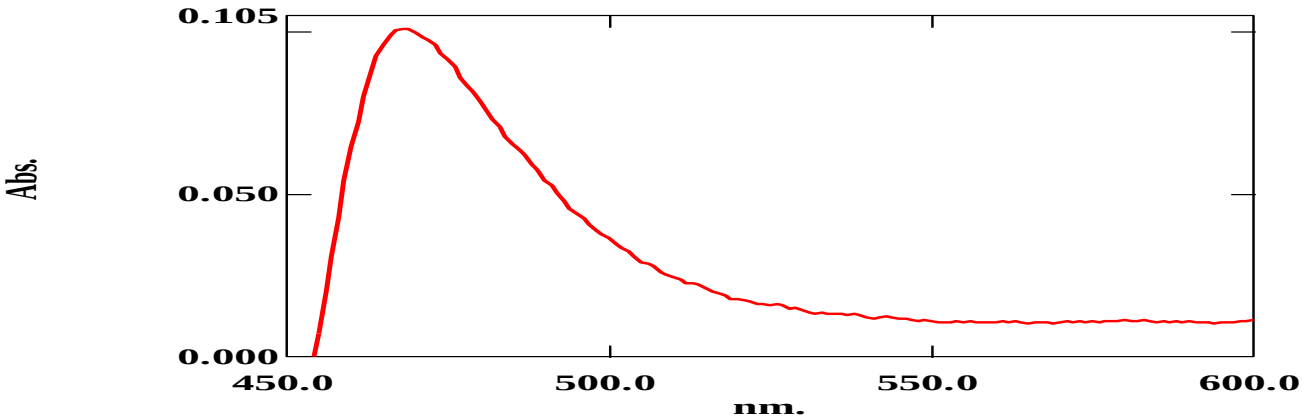


Figure 2: Absorption spectra of the reaction product of 50 µg.mL^{-1} FA vs, under primary test

Optimization of Experimental Conditions

The optimum experimental conditions were studied by two different approaches, the univariate and the multivariate. Different parameters affecting the color producing reaction i.e. the type and the amounts of Noah, H_2SO_4, $NaNO_2$, coupling reagent, reaction temperature, heating time, and the type and the amounts of the base, were carefully studied following one factor a time univariate methodology.

The results show that among different acids (HCl, HNO_3, H_2SO_4.CH_3COOH and H_3PO_4) used as a medium for diazotization of the studied drug, 0.7 mL of 1.85 M sulfuric acid solution in the presence of 0.2mL 4 %(w/v) sodium nitrite solution gave the best result when the flasks were maintained in an ice bath (0-5) C for 5min (Fig. 3, 4, and 5).

On the other hand, it was found that using 0.4mL of 2% (w/v) of 4-amino antipyrine solution as a coupling reagent gave the maximum color intensity when the reaction was allowed to stand in a water bath for 1 minute at 85 C (Fig. 4). In general, to arrange the coupling between the diazotized drug and the coupling reagent in the favors of coupling rather than the diazo decomposition, the value of pH and temperature of the coupling medium must be optimized since this type of reaction is very sensitive toward them [22].

Accordingly, different volumes of different alkalis (Noah, KOH, LiOH.H_2O, and NH_4OH) solutions were tried and among them, 1mL of 4M KOH solution was selected since it resulted in the highest value of absorption (Fig. 3, and 5) when the reaction medium was left for 30 sec. in an ice bath before dilution to the final volume with distilled water.

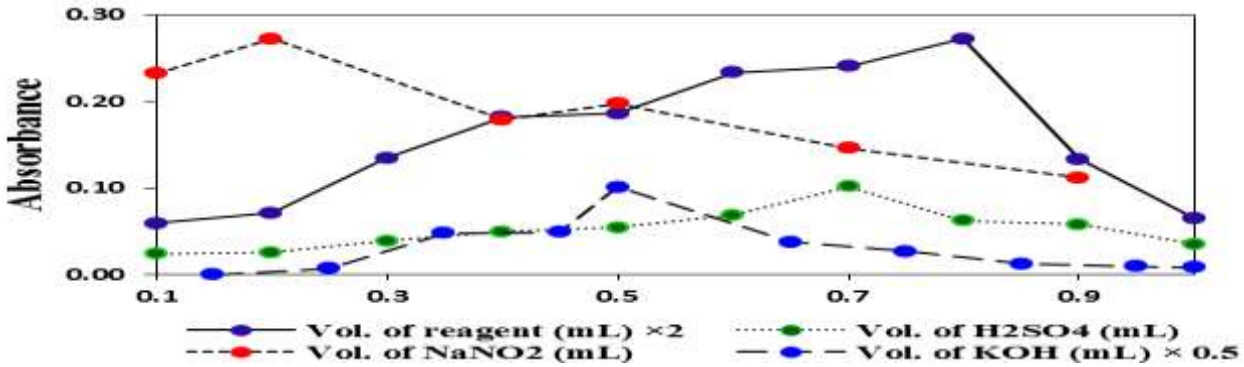


Figure 3: The effect of H_2SO_4, $NaNO_2$, 4-aminoantipyrine, and KOH on the reaction product

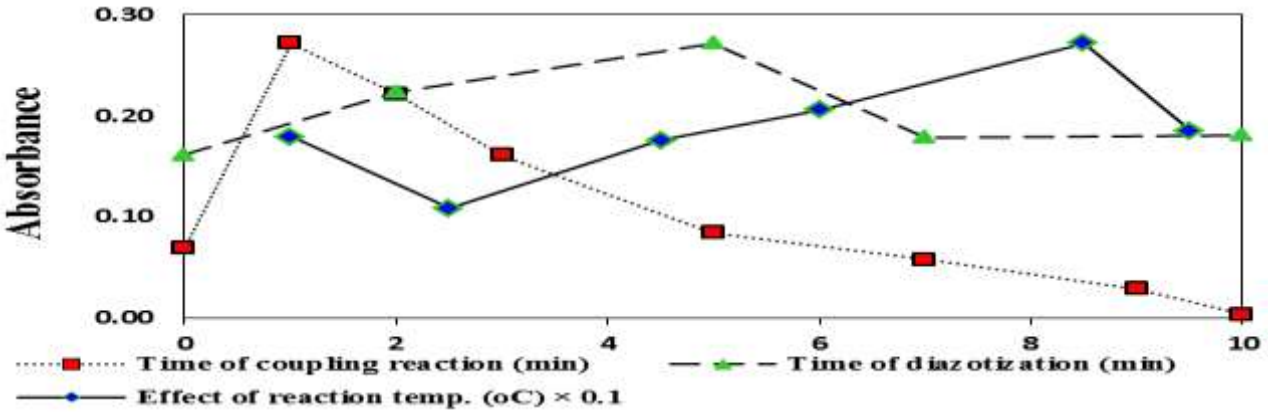


Figure 4: The effect of the time of diazotization, time of coupling, and reaction temperature on the reaction product

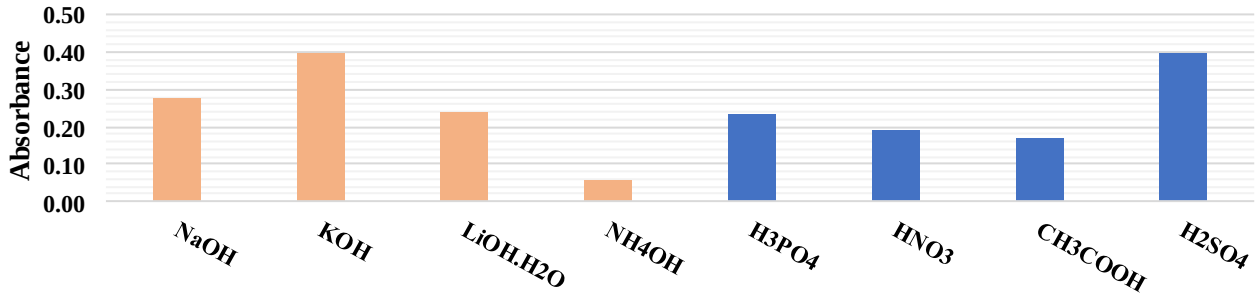


Figure 5: Effect of the type of acid and base on the reaction product

Over and above, multivariate experimental design procedure was followed to study the optimum values of the most important factors affecting the formation of the colored azo-dye namely the volume of 4M KOH solution, the volume of 2%(w/v) of 4-amino antipyrine solution and the heating time. Table 1 shows the face-centered central composite design (FCCD) matrix as well as the experimental results. The experiment corresponding to the central point accomplished in four repeats. The measurements were accomplished with 50 µg.mL^{-1} solution of folic acid.

Table 1: Matrix and results of the central composite design

Exp. No.	Factors			Absorbance (469 nm)
	(X_1)	(X_2)	(X_3)	
	Vol. of KOH	Vol. of Reagent	Heating time	
1	0.3	0.05	0	0.003
2	0.3	0.05	10	0.000
3	0.3	0.55	0	0.018
4	0.3	0.55	10	0.012
5	2.1	0.05	0	0.032
6	2.1	0.05	10	0.021
7	2.1	0.55	0	0.010
8	2.1	0.55	10	0.038
9	0.3	0.30	5	0.008
10	2.1	0.30	5	0.101
11	1.2	0.05	5	0.035
12	1.2	0.55	5	0.078
13	1.2	0.30	0	0.262
14	1.2	0.30	10	0.050
15©	1.2	0.30	5	0.087
16©	1.2	0.30	5	0.087
17©	1.2	0.30	5	0.087
18©	1.2	0.30	5	0.087
19©	1.2	0.30	5	0.087
20©	1.2	0.30	5	0.087

The experimental data were fitted using a linear-quadratic second-order polynomial main effects model with following equation:

$$Abs. = (-0.0571) + (0.1981\ X_1) + (-0.0744\ X_1^2) + (0.5883\ X_2) + (-0.9316\ X_2^2) + (-0.0225\ X_3) + (0.0017\ X_3^2) + (-0.0178\ X_1X_2) + (0.0007\ X_1X_3) + (0.0036\ X_2X_3)$$

Accordingly, the predicted optimum values of the studied factors are: volume of 4M KOH solution is 1.3243mL (≈ 1.32 mL), volume of

reagent solution is 0.3151 mL (≈ 0.32 mL), and the heating time is 6.1921 min. (≈ 372 sec.). The relationship of absorbance against the investigated factors are represented in three three-dimensional response surface plots, Figure 6.

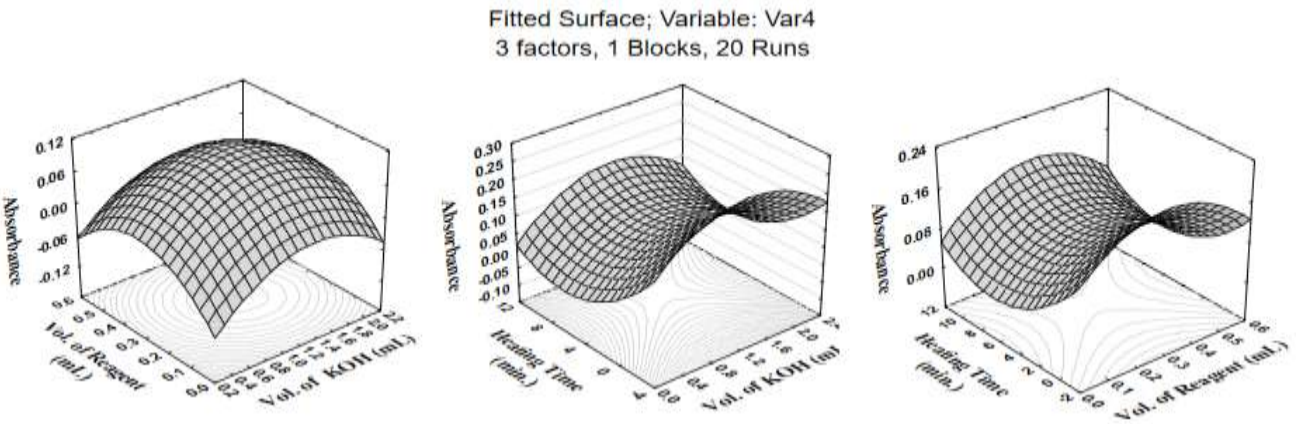


Figure 6: Response surface of quadratic model for absorbance values as a function of KOH solution volume, reagent volume, and heating time

Effect of Order Addition

Different orders were used to mix the solutions of the reagents used in this investigation. It was found that the addition of the drug solution to the reagent solution followed by adding the base solution (the first order in Figure 7) gave the maximum absorbance. On the other hand, following other sequences result in a loss of color intensity. Therefore, this order was recommended for the subsequent experiments.

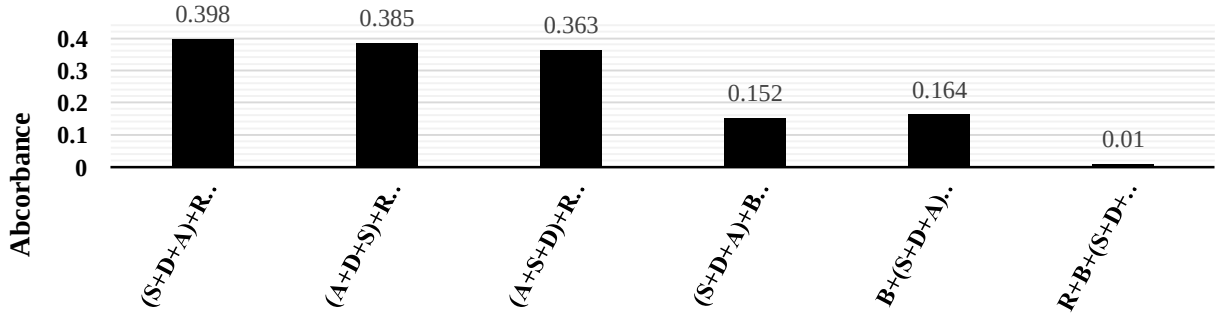


Figure 7: Effect of mixing order on the absorbance of the azo dye, (*D: Drug, R: Reagent, B: Base, A: Acid, S: Salt*)

Stability

The effect of time on the formed product was investigated by allowing it to stand for varying periods. The maximum color intensity is reached after mixing the components of the reaction, and the absorbance of the formed dye remained constant for at least 60 minutes. Fig. 8.

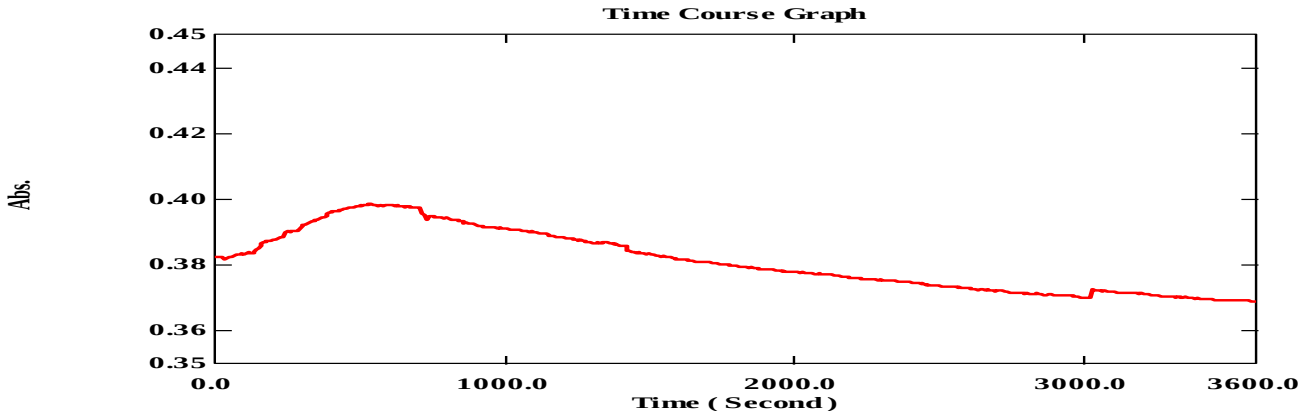


Fig.8: Effect of time on color development

Final Absorption Spectrum

Figure 9 shows the final spectrum of the azo dye product formed exhibits a maximum at 469 nm, under the optimum conditions established with univariate and multivariate.

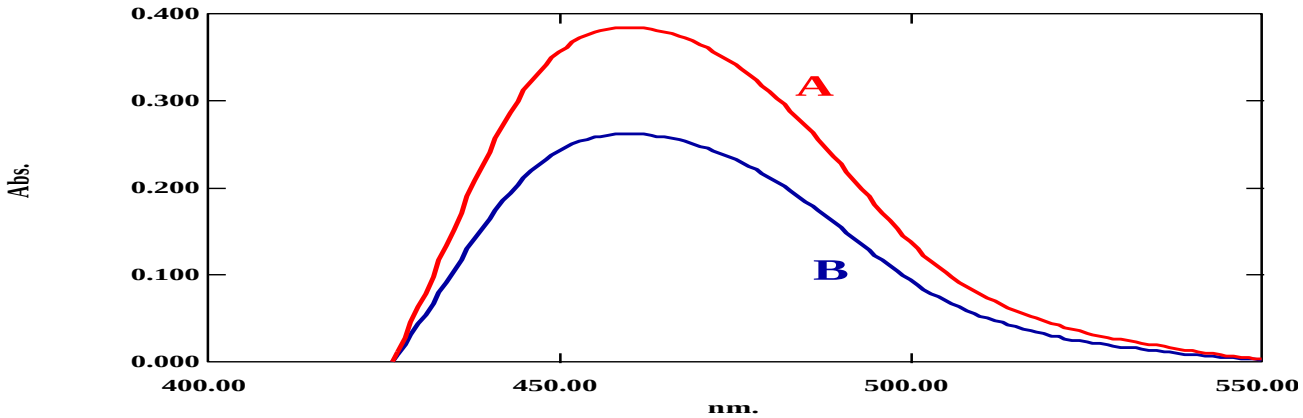


Figure 9: The absorption spectrum of the reaction product of 50 µg. mL^{-1} FA vs reagent blank, (A)under the univariate conditions, (B) multivariate conditions

Validation of Beer's Law (Linearity, Accuracy, and Precision)

The value of the measured absorbance, at 469 nm, of the azo dye formed under the univariate and multivariate experimental design conditions, was found to be in a linear relationship with the concentration of folic acid in the range of 2-100 µg. mL^{-1}. The values of the molar absorptivity were 3266.36 L. mol^{-1}.cm^{-1} and 2471.84 L. mol^{-1}.cm^{-1} when the calibration curves (Figure 10) were constructed under experimental conditions obtained by univariate and multivariate methodology. Table (2) shows other quantitative and statistical parameters for the determination of folic acid using the proposed method. Moreover, the accuracy and precision of the determination of folic acid via the proposed method were studied by calculating the values of percentage of the relative standard deviation percentage (RSD %) and the percentage of relative error (RE %), for three replicates at three different concentration levels of FA. The results in Table 3 show acceptable values for accuracy and precision which were obtained.

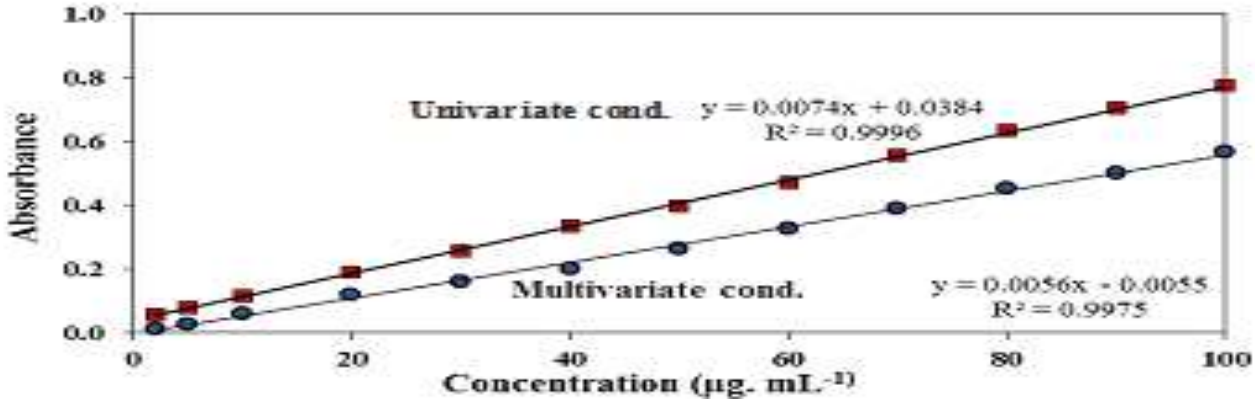


Figure10: Calibration graphs of FA constructed under univariate and central composite design experimental conditions

Table 2: Optical characteristics, statistical data of the regression equations and validation parameters of FA

Parameter	Value	
	Univariate	CCD
Optical characteristics		
1. λ_{max} (nm) **2.** Apparent molar absorptivity (L.mol^{-1}.cm^{-1}) **3.** Sandell's sensitivity (µg.cm^{-2}) **4.**	469.00 3266.36 0.1351	467.00 2471.84 0.178 6
Regression analysis		
1. Slope (L. mg^{-1}.cm^{-1}) **2.** Intercept **3.** Regression coefficient (r)	0.0074 0.0384 0.9983	0.0056 0.0055 0.9987

Validation parameters		
1. Beer's Law Limit (Linearity, µg. mL^{-1})	2-100	2-100
2. Limit of detection (µg. mL^{-1})	0.1482	0.6056
3. Limit of quantitation (µg. mL^{-1})	0.4941	2.0186

Table 3: Evaluation of accuracy and precision

Conc. of FA (µg.mL^{-1})			*RE %	RSD %
Method	**Taken**	**Found***		
Univariate	15	14.8739	-0.8408	0.0139
	35	34.1824	-2.3359	0.0099
	60	58.6126	-2.3123	0.0235
FCCD	10	10.0060	0.0595	0.9158
	30	29.5089	-1.6369	0.8537
	60	59.9792	-0.0347	0.3451

*Average of three measurements

Stoichiometry of the Formed Product

The stoichiometric ratio of the reactants was determined by employing Job's method of continuous variation [29] and the molar ratio method [30]. The results indicated that the interaction occurs between equimolar solutions of FA and reagent and the compound was formed in the ratio of 1:1 as illustrated in Figures (11 and 12) and Scheme 1.

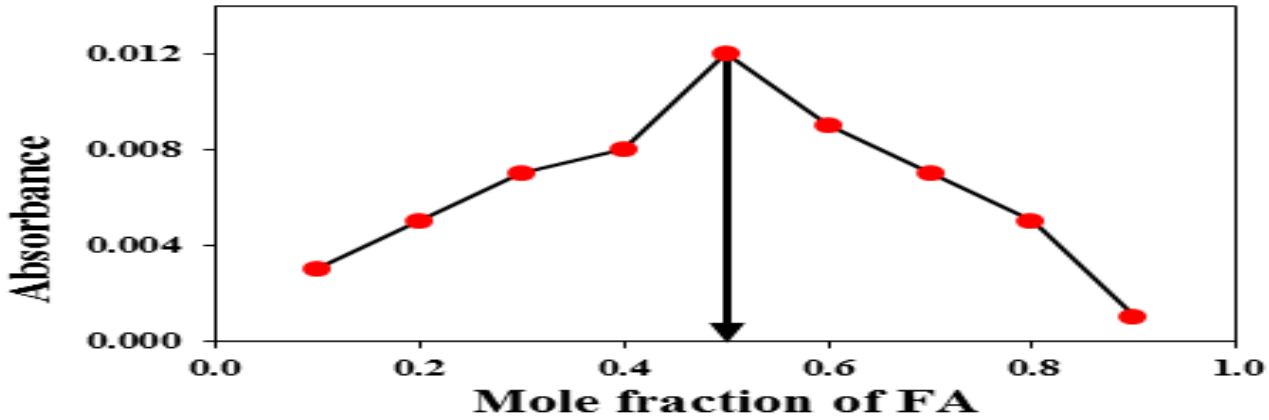


Figure 11: Job's method of continuous variation

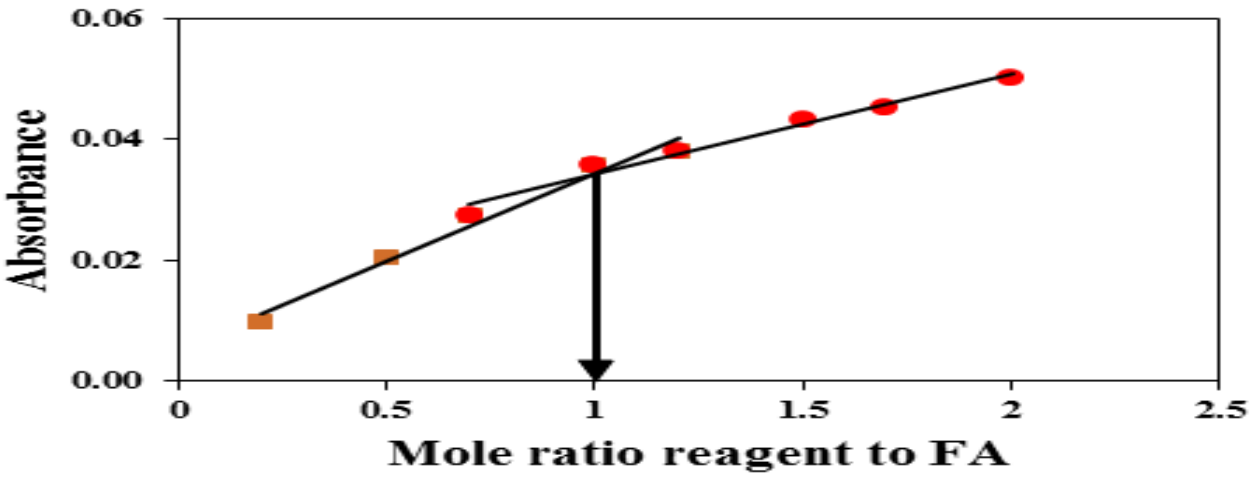
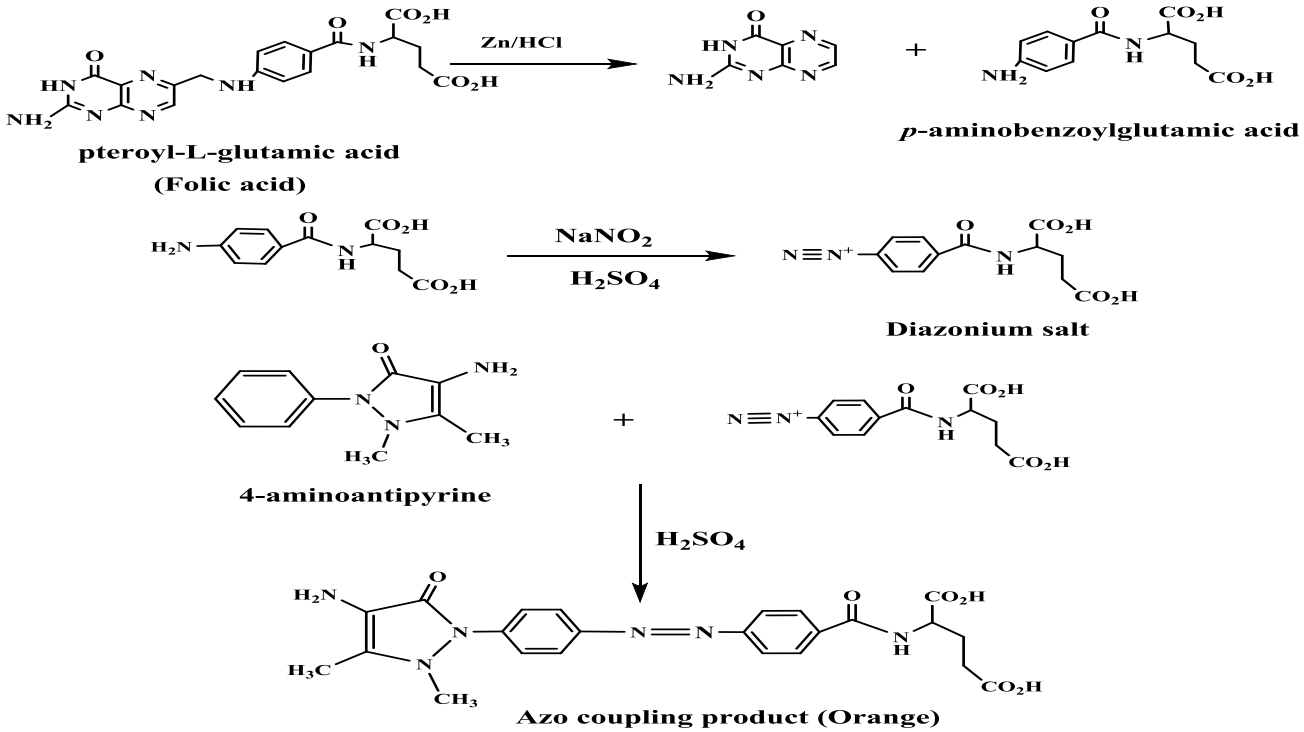


Figure 12: Mole ratio method

Accordingly, the mechanism of diazotization of folic acid and the coupling of the diazotized compound with 4-aminoantipyrine can be suggested as in Scheme 1.



Scheme 1: The suggested steps of the formation of the colored azo-dye

Interference Studies

To assess the analytical potential of the proposed method, the effect of some common excipients; sucrose, glucose, lactose, talc, cellulose, Mg stearate, Na-stearate, and acacia, were examined by carrying out the determination of 50 µg. mL^{-1} of FA in the presence of (1000 µg. mL^{-1}) above compounds. The results are presented in Table 4.

Table 4: Percent recovery for 50 µg.mL^{-1} of FA in the presence of excipient

Excipients	Con. Found µg.mL^{-1}	%Recovery
Sucrose	49.0730	98.14595
Glucose	47.5676	95.13514
Lactose	49.5091	99.01811
Talc	49.3543	98.70865
Cellulose	49.4810	98.96189
Mg stearate	48.1726	96.34514
Na stearate	48.3976	96.79514
Acacia	49.4246	98.84919

Application in Pharmaceutical Preparation

For verifying the efficiency of the proposed method, it was applied on real samples with known contents of FA i.e. tablet (containing 5 mg FA/tablet). The results of the application are given in Table 5 were satisfactory.

Table 5: Analysis of FA in the pharmaceutical tablet

Sample	Conc. of FA (µg.mL^{-1})		*Recovery %	*RSD%
	Taken	*Found		
S.D.I	10	10.0270	100.2703	2.3691
	30	29.4820	98.2733	0.4901
	50	48.7027	97.4054	0.1272
MED (Lebanon)	10	9.7387	97.3874	0.8131
	30	29.6351	98.7838	1.4282
	50	48.7973	97.5946	0.0999
Fulcium (UAE)	10	10.3243	103.2432	0.9439
	30	29.3108	97.7027	0.1597
	50	49.2523	98.5045	1.9172
Folic guard (Bulgaria)	10	9.6577	96.5766	0.6463
	30	29.2658	97.5526	0.2324

	50	49.8604	99.7207	1.3590
Folic acid (U.K).	10	9.8919	98.9189	0.5955
	30	30.0045	100.0150	0.2896
	50	50.0451	100.0901	0.5266

*Average of three measurements

Conclusion

The proposed method is simple and sensitive with reasonable accuracy and precision. The method suffers no interference from the common additives and excipients and was applied successfully for the determination of folic acid in its pure form or in pharmaceutical preparations.

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